MORPHOLOGY AND TOXICITY CHARACTERISTICS OF CYANOBACTERIA IN EAST MALAYSIA

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INTRODUCTION

- Cyanobacteria
  - Unicellular
  - Colonial
  - Filamentous
  - Heterocysts (nitrogen fixation)
  - Akinetes (resting cells)
INTRODUCTION (cont.)
<table>
<thead>
<tr>
<th>General features</th>
<th>Toxin Group</th>
<th>Primary Target Organ</th>
<th>Cyanobacterial genera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cyclic Peptides</strong></td>
<td>Microcystin</td>
<td>Liver</td>
<td>Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis, Nodularia</td>
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<tr>
<td></td>
<td>Nodularian</td>
<td>Liver</td>
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<tr>
<td><strong>Alkaloids</strong></td>
<td>Anatoxin-a</td>
<td>Nerve synapse</td>
<td>Anabaena, Planktothrix (Oscillatoria), Aphanizomenon</td>
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<td>Anatoxin-a(S)</td>
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<td>Anabaena</td>
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<td>Skin</td>
<td>Lyngbya, Schizothrix, Planktothrix (Oscillatoria)</td>
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<td>Cylindrospermopsins</td>
<td>Liver</td>
<td>Cylindrospermopsis, Aphanizomenon, Umezakla</td>
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<td>Lyngbyatoxin-a</td>
<td>Skin, G.I. Tract</td>
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<td>Saxitoxins</td>
<td>Nerve Axons</td>
<td>Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis</td>
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<tr>
<td><strong>Lipopolysaccharides</strong></td>
<td>(LPS)</td>
<td>Potential irritant, Affects any exposed tissue</td>
<td>ALL</td>
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</tbody>
</table>
Background

- Cyanotoxins are produced by several species of cyanobacteria (cyanobacteria are known as blue-green algae).
- The most widespread of the cyanotoxins are the peptide toxins in the class called microcystins.
- There are at least 80 known microcystins, including Microcystin-LR, which is generally considered one of the most toxic.
- World Health Organization provisional value for drinking waters of 1.0 µg/L microcystin-LR
Table 1. Cyanotoxins on the Contaminant Candidate List (CCL)

<table>
<thead>
<tr>
<th>Cyanotoxin</th>
<th>Number of known variants or analogues</th>
<th>Primary organ affected</th>
<th>Health Effects&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Most common Cyanobacteria producing toxin&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystin-LR</td>
<td>80–90</td>
<td>Liver</td>
<td>Abdominal pain&lt;br&gt;Vomiting and diarrhea&lt;br&gt;Liver inflammation and hemorrhage</td>
<td>Microcystis&lt;br&gt;Anabaena&lt;br&gt;Planktothrix&lt;br&gt;Anabaenopsis&lt;br&gt;Aphanizomenon</td>
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<tr>
<td>Cylindrospermopsin</td>
<td>3</td>
<td>Liver</td>
<td>Acute pneumonia&lt;br&gt;Acute dermatitis&lt;br&gt;Kidney damage&lt;br&gt;Potential tumor growth promotion</td>
<td>Cylindrospermopsis&lt;br&gt;Anabaenopsis&lt;br&gt;Lyngbya&lt;br&gt;Rhaphidiopsis&lt;br&gt;Umezakia</td>
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<tr>
<td>Anatoxin-a group&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2-6</td>
<td>Nervous System</td>
<td>Tingling, burning, numbness, drowsiness, incoherent speech, salivation, respiratory paralysis leading to death</td>
<td>Anabaena&lt;br&gt;Planktothrix&lt;br&gt;Aphanizomenon&lt;br&gt;Cylindrospermopsis&lt;br&gt;Oscillatoria</td>
</tr>
</tbody>
</table>

<sup>1</sup>Source: Harmful Algal Research and Response National Environmental Science Strategy (HARRNESS)

<sup>2</sup>Not all species of the listed genera produce toxin; in addition, listed genera are not equally as important in producing cyanotoxins.

<sup>3</sup>The anatoxin-a group does not include the organophosphate toxin anatoxin-a(S) as it is a separate group. In the US, the most common member is thought to be anatoxin-a, and thus this toxin is listed specifically.
Risk of exposure to cyanotoxins

- drinking water
- recreational water
- dietary supplements
- residue on produce irrigated with contaminated water and consumption of animal tissue
How you could be exposed to cyanotoxins?

1. Drinking water that comes from a lake or reservoir
2. Drinking untreated water
3. Doing recreational activities in waters with harmful blue-green algae blooms (cyanobacteria)
4. Inhaling aerosols from water-related activities such as jet-skiing or boating
5. Inhaling aerosols when watering lawns, irrigating golf-courses, etc. with pond water
6. Using blue-green algae based dietary supplements (if they are contaminated with microcystins)
7. Having dialysis (this has only been documented in Brazil)
LIVER FAILURE AND DEATH AFTER EXPOSURE TO MICROCYSTINS AT A HEMODIALYSIS CENTER IN BRAZIL

ABSTRACT

**Background** Hemodialysis is a common but potentially hazardous procedure. From February 17 to 20, 1996, 116 of 130 patients (89 percent) at a dialysis center (dialysis center A) in Caruaru, Brazil, had visual disturbances, nausea, and vomiting associated with hemodialysis. By March 24, 26 of the patients had died of acute liver failure.

**Methods** A case patient was defined as any patient undergoing dialysis at dialysis center A or Caru-
Results  One hundred one patients (all at dialysis center A) met the case definition, and 50 died. Affected patients who died were older than those who survived (median age, 47 vs. 35 years; P<0.001). Furthermore, all 17 patients undergoing dialysis on the Tuesday-, Thursday-, and Saturday-night schedule became ill, and 13 of them (76 percent) died. Both centers received water from a nearby reservoir. However, the water supplied to dialysis center B was treated, filtered, and chlorinated, whereas the water supplied to dialysis center A was not. Microcystins produced by cyanobacteria were detected in water from the reservoir and from dialysis center A and in serum and liver tissue of case patients.

Figure 2. System of Water Treatment and Distribution in Caruaru, Brazil.
Observational Studies

Tabocas Reservoir is located approximately 40 km from Caruaru. A pipeline carries water from the reservoir to the municipal water-treatment plant (Fig. 2). There, alum was added to the water. After settling for two to three hours, the water was filtered through a large-particle sand filter, and then chlorine was added. This “finished” water was distributed through a water-distribution system to most of Caruaru, including dialysis center B. Dialysis center A was not included in this water-distribution system during the 1996 summer drought. Instead, dialysis center A received “unfinished” water, trucked from the municipal water-treatment plant twice daily. This water was treated with alum but not filtered or chlorinated. Occasionally, personnel at the water-treatment plant gave the truck driver chlorine to add to the water in his truck. However, there are no records to indicate whether or when chlorine was added during February and March 1996. After arriving at dialysis center A, the water was passed through a sand filter, a carbon-adsorption tank, a deionizer unit comprising cation and anion tanks, and a micropore filter before being used for hemodialysis. No chemicals were added to the water at the dialysis center. According to maintenance workers at dialysis center A, the carbon tank was changed approximately every six months, and the sand and micropore filters were changed approximately every three months; however, these devices had not been changed in the three months before the probable exposure, even though the center was receiving visibly turbid water from the delivery truck. On Saturday, February 17, 1996, after patients first reported symptoms, the sand and micropore filters at dialysis center A were changed. The carbon in the adsorption tank was changed on February 25, 1996.
WHY CYANOBACTERIA?

1. LIMITED STUDY IN SABAH AND SARAWAK

MORPHOLOGY?

2. LESS INFORMATION ABOUT SPECIES DESCRIPTION ESPECIALLY IN BORNEO ISLAND

TOXICITY?

3. LACK OF TOXICITY STUDY

4. MONITORING FOR PUBLIC SAFETY
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<th>NO.</th>
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<th>DISTRIBUTION SYSTEM</th>
<th>WELL/SPRING</th>
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NATIONAL STANDARD FOR DRINKING WATER QUALITY

- prepared by -
Engineering Services Division
Ministry of Health Malaysia

Revised December 2000
Second Revision, January 2004
Objectives

- To describe morphological characteristics of cyanobacteria isolated for pond
  - Inverted Light Microscope
  - Scanning Electron Microscopy

- To determine toxicity of potential toxic species
  - Brine shrimp assay/Mouse bioassay (crude extraction)
  - Thin layer chromatography/High performance liquid chromatography (partially purified toxin)
FRGS/06(02)/657/2007(23)
Diversity, Phylogeny and Toxins of Freshwater Cyanobacteria in Selected Aquaculture Ponds

Sampling Locations
Indigenous Fisheries Research and Production Centre, Kuching, SARAWAK

(a) Earth pond (P1)  
(b) HDPE (AP12)  
High Density Polyethylene
Babagon Fisheries Centre, SABAH

Earth pond
Samples collection, cell isolation, species ID,

- Samples (cyanobacteria) were collected by using plankton net with **20µm** mesh size.
- **Cell isolation** were done in laboratory using *Inverted Light Microscope* (Olympus M1025- Microscope Research Fluorence Model 1X51RFLCCD).
- **Species identifications** were based on keys according to Anagnostidis and Komarek (1985, 1988); Komarek and Anagnostidis (1986, 1989); Holt et al., (1994); Bold and Wayne (1985); Prescott (1962); Sze (1993) and credible supplementary online materials.
Species ID

- Inverted Light Microscope
  (*Olympus M1025*-Microscope Research Fluorescence Model 1X51RFLCCD)

- Analytical Scanning Electron Microscopy
  (*JEOL JSM-6390LA*).
SEM

1. **Primary fixation** - 5% glutaraldehyde fixative
2. Then, **washed** with 0.1M cacodylate buffer prior to secondary fixation
3. Samples were **post fixed** in 1% osmium solution
4. After incubation for 1 hour in room temperature, the **fixative was discarded** and samples were **rinsed** with 0.1M cacodylate buffer and ready for dehydration process.
5. **Prior dehydration**, **cells were transferred into polycarbonate membrane by mild filtration using vacuum manifold.**
6. Algal filaments (filter papers) were **dehydrated in ethanol concentration series of 10%, 20%, 40%, 60%, 80%, 90%, 95% and 100%.**
7. Then, samples were **dried** (critical point drying) with **Critical Point Dryer SEM (CPD 030 Bal Tec)** and it was **coated with gold palladium** using **Auto Fine Coater SEM (JEOL JFC-1600).**
### RESULTS (FIELD SAMPLES – ISOLATED)

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Order</th>
<th>Genera (Strain)</th>
</tr>
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<tbody>
<tr>
<td>IFRPC, Sarawak</td>
<td>Chroococcales</td>
<td><em>Microcystis</em> (MIC1, MIC2, MIC3)</td>
</tr>
<tr>
<td></td>
<td>Oscillatoriales</td>
<td><em>Oscillatoria</em> (OSC1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lyngbya</em> (LYN1)</td>
</tr>
<tr>
<td></td>
<td>Nostocales</td>
<td><em>Anabaena</em> (ANA1, ANA2, ANA3, ANA4, ANA5)</td>
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<td></td>
<td><em>Cylindrospermopsis</em> (CYL1)</td>
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<tr>
<td>BFC, Sabah</td>
<td>Nostocales</td>
<td><em>Nostoc</em> (NOS1)</td>
</tr>
</tbody>
</table>
**Chroococcales**

*Microcystis aeruginosa* strain MIC1 isolated from IFRPC Tarat [100x magnification]

Scanning electron micrograph of MIC1 (cell colony) and (binary fission) of cells.

*Microcystis sp* strain MIC2

*Microcystis flos-aquae* (eg. Lack of slimy margin of colony’s edge)

*Microcystis sp* strain MIC3

*Microcystis botris* (cells size were mostly larger than 3 µm)

Cells were **oval to spherical** with diameter range of 3±0.05 µm (mostly >3µm), binary fission in three planes and irregularly oriented in **aggregates**.
Oscillatoriales

Oscillatoria sp. (OSC1)

Trichomes sheathless, dark blue-green in color, nearly straight, constricted at cross walls, attenuated at the apex, and bent.

Lyngbya sp. (LYN1)
Nostocales

Anabaena

- Trichomes - irregularly coiled, solitary
- Heterocysts - spherical to elliptical
- Akinetes - cylindrical or sausage-shaped
Nostocales

Cylindrospermopsis sp. CYL1

Trichomes - coiled, vegetative cells with well-developed aerotpoes were variable in length, but always longer than wide.

Vegetative cells - generally cylindrical and their size varied from 2.78-4.98 µm in width and 3.87-12.54 in length.

Nostoc sp. NOS1

Trichomes - untapered with conspicuous constriction at cross-walls, isopolar, same width along the whole trichome and apical cells were morphologically similar with other cells.

Vegetative cells - cylindrical, spherical or ovoid (barrel-shaped) and not shorter than broad with the size varied from 5-6 µm in diameter.
(TOXICITY CHARACTERISTICS)

Biological Assay – Mouse bioassay and Brine shrimp assay
Analytical Analysis – TLC and HPLC
Figure 13.1 Relationship between sensitivity and selectivity of analytical methods for microcystins
Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management (Edited by Ingrid Chorus and Jamie Bartram)

Chapter 13. LABORATORY ANALYSIS OF CYANOTOXINS

www.who.int/water_sanitation_health/.../toxcyanchap13.pdf
Toxin extraction

Harada et al. (1999)

Figure 13.2 Toxin extraction by filtration

- Sample filtering
- Filter is thawed and extracted
- Extract is analysed by HPLC

- Wet filter is frozen at -20 °C
- Dried filter and some filtrate are mailed to laboratory for toxin determination
- Filtrate is analysed by HPLC for extracellular toxin
- Dried filter is rehydrated with water

OR

- Filter is thawed and extracted
- Extract is analysed by HPLC
MASS CULTURE

Harvested at exponential phase (BG11 Medium, 25°C, 10 µmol·m⁻²·s⁻¹ irradiance)

Samples (cyanobacteria cells)
  Freeze-dried

Lyophilized cells
  Extracted with MeOH

Crude extract (Test solution)

- Brine shrimp assay
- Mouse bioassay
- TLC
- HPLC
The freeze-dried cyanobacteria material was extracted with methanol and evaporated to dryness in a vacuum.

The dried extract was dissolved in 6ml filtered seawater to give a concentration as 1mg/ml in terms of lyophilized cell masses and further diluted with seawater to give five concentrations of 100µg/ml, 50µg/ml, 20µg/ml, 10µg/ml, and 2µg/ml.

Assays were performed on a 24-well plate with 10 brine shrimp larvae per well tested.

The brine shrimp were observed for 24 hours to calculate the mortality.
RESULTS
Mouse bioassay

Lyophilized cells of cultured cyanobacteria were extracted three times with 10 ml methanol and further dried in vacuum.

Dried extract from cyanobacteria were re-dissolved in sterile 0.9% NaCl solution (normal saline) in different concentrations.

Toxicity assay was done by using three mice tested for each dose level.

One milliliter of the extract was injected intraperitoneally (i.p.) to mice weighing 20 g each.

Symptoms were observed until 4 hours for lethal results and following 48 hours for total observation.
Relationship between injected dose of *Microcystin* sp. (strain MIC1) extract and LT$_{100}$ (time needed to observe death response after injection) of mice.
Relationship between injected dose of Anabaena spp. (strain ANA1) extract and LT$_{100}$ (time needed to observe death response after injection) of mice.
Relationship between injected dose of Nostoc sp. (strain NOS1) extract and LT$_{100}$ (time needed to observe death response after injection) of mice.
*Lyngbya* sp. (LYN1)  
*Cylindrospermopsis* sp. (CYL1)  

-ve results for mouse bioassay
Thin Layer Chromatography (TLC)

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<th>Samples</th>
<th>Rf values</th>
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<tr>
<td>ANA 1</td>
<td>0.96</td>
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*Microcystin-LR standard (Enzo Life Sciences, Ltd, UK)

Mobile phase: chloroform:methanol:water (65:35:10 v/v, lower phase)
High Performance Liquid Chromatography (HPLC)

LYN 1

CYL 1

ANA4

NOS1
FINDINGS

Cyanobacteria (isolated/identified)

3 Orders, 6 Species
- Microcystis (MIC 1-3)
- Oscillatoria (OSC1)
- Lyngbya (LYN1)
- Anabaena (ANA1-5)
- Cylindrospermopsis (CYL1)
- Nostoc (NOS1)

Brine shrimp assay

Dose-dependent response in mortality of selected strains (MIC1, ANA1, ANA4, NOS1, LYN1, CYL1) were observed.

Mouse bioassay

Microcystis > Anabaena > Nostoc
Cylindrospermopsis & Lyngbya (-ve results)
FINDINGS (cont.)

TLC

+ve results (MIC 1, ANA 1) – same or almost identical Rf values with MC-LR standard

HPLC

+ve results (MIC1, ANA1) - based on retention time comparison
-ve results (ANA4, LYN1, CYL1, NOS1)
CONCLUSION

MIC 1 (Sarawak)

+ve results in brine shrimp assay, mouse bioassay, TLC and HPLC analysis (Potential toxic species)

ANA1 (Sarawak) & NOS1 (Sabah)

+ve results in brine shrimp assay & mouse bioassay
ANA1 – TLC & HPLC
REFERENCES


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Toxicity Assessment of Cyanobacterial Strains Using Brine Shrimp and Mouse Bioassay

Morphological Study and Toxicity Assessment of Cultured Cyanobacteria from Fish Pond
HUMAN CAPITAL DEVELOPMENTS

- Lai Jia Rhou (Undergraduate student)  
  Toxicity Assessment of Cultured Cyanobacteria Toxin by Using Bioassay (Brine Shrimp) and TLC and HPLC” (2010-2011)

- Jasmina Majit (MSc student)  
  Morphology and Toxin Properties of Clonal Culture Cyanobacteria from selected Aquaculture Ponds (2009-2012)